STUDIES ON URINARY PHENOLIC ACID METABOLITES OF NOREPINEPHRINE

by

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A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Master of Arts

Department of Biological Chemistry

University of Utah

June 1958

This Thesis for the M. A. degree

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has been approved by

Reader, Supervisory Committee

Reader, Supervisory Committee

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ACKNOWLEDGEMENTS

The author is sincerely grateful to Dr. M. D. Armstrong for suggesting the problem in this thesis and for his guidance and supervision in the course of the ensuing research.

The assistance and enthusiasm of Dr. K. N. F. Shaw is also deeply appreciated.

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INTRODUCTION

Norepinephrine is a very important biologically active compound by virtue of its physiological functions as a sympathetic neurohormone and a suprarenal medullary hormone. The realization of its importance however, came relatively late in the course of its history.

Norepinephrine was first synthesized more than 50 years ago because of its structural similarity with epinephrine, a hormone of the adrenal medulla which had already been isolated in crystalline form from tissues. Then, except for an occasional testing of its pharmacological properties, norepinephrine remained a laboratory curiosity for about 40 years.

During this period of relative obscurity, physiologists discovered and studied new distinct physiological activities which were ascribed to substances called sympathin E and sympathin I; the potent physiological properties of norepinephrine were established concurrently, and the absence of such a logical compound from the body of animals came to be regretted to the point that nature was suspected of inefficiency. Finally, after arterenol was isolated from several tissues, proof was obtained that norepinephrine, sympathin E and arterenol are the same compound.

The wide biological distribution and the striking physiological properties so suddenly inherited by norepinephrine from its synonyms sparked the interest of literally hundreds of workers with the result

that more than 500 papers have been published about the compound in the last ten years. This gigantic effort has dealt with all aspects of norepinephrine, namely distribution, determination, chemistry, physiology, pharmacology and metabolism.

This thesis is concerned with the metabolism of norepinephrine. A brief survey of the pertinent literature is presented in order to place the present study in proper historical perspective. Evidence is offered which demonstrates that 3-methoxy-4-hydroxymandelic acid is an important metabolite of norepinephrine.

REVIEW OF THE LITERATURE

Norepinephrine is one of the sympathomimetic amines, a group of compounds of great physiological importance. It has been known variously under the names of noradrenaline NOR:(Stickstoff) - N - Ohne Radikal , sympathin E and arterenol.

The Discovery of Norepinephrine

Norepinephrine, α-3,4-dihydroxyphenyl-β-aminoethanol, was first synthesized in 1904 by Stolz (1) who also made the first observations on its biological properties. Two years later Biberfeld (2) showed that the compound is as active as epinephrine in raising the blood pressure of animals and first suggested a clinical use for it. It was only in 1948, however, that norepinephrine was shown by von Euler (3) to be the specific action substance of adrenergic nerves. Holtz, Credner and Kroneberg (4) reported also at that time that urine contains what appeared to be conjugated norepinephrine and that extracts of mammalian adrenals seem to contain the same substance.

In the period which elapsed between 1904 and 1948, norepinephrine was neglected in favor of its homologue, epinephrine; very little work was done with the compound, and dealt mainly with its pharmacological properties. Nerve experiments by Cannon and collaborators (5, 6) led these workers to suspect that the stimulation of sympathetic nerves caused the release of a substance other than epinephrine; they called this sub-

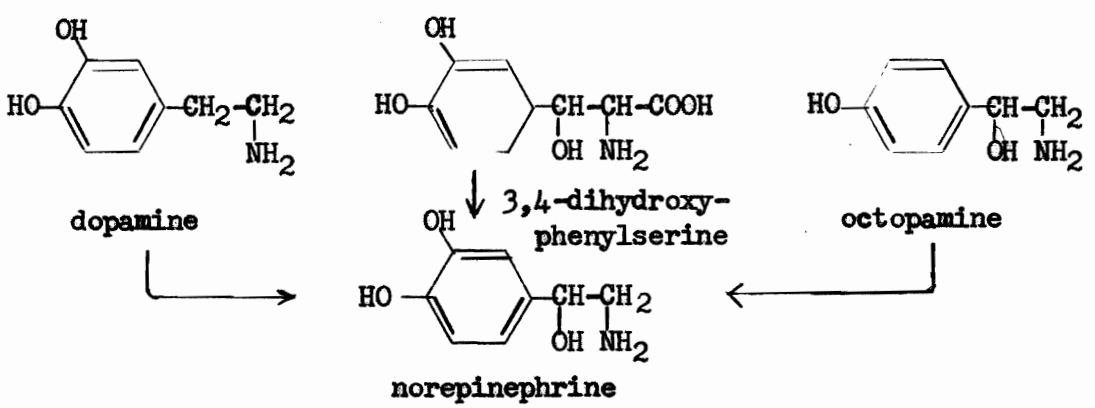
stance sympathin E. In 1934, Bacq (7) proposed that sympathin E and norepinephrine were the same entity. Much experimental evidence that followed has reinforced this concept. It remained for von Euler (3, 8) to show that extracts of adrenergic nerves and of organs supplied by them contain appreciable quantities of arterenol, specifically the levo form, and that this compound is norepinephrine.

The Chemistry, Physiology and Pharmacology of Norepinephrine

These subjects have been extensively reviewed recently in a comprehensive monograph by von Euler (9) and it is beyond the scope of the present work to elaborate on them. Suffice it to say that norepinephrine causes an impressive increase in the peripheral vascular resistance of animals, augmenting thereby both the systolic and diastolic blood pressure; it has very little influence on cardiac output and the dose required to affect carbohydrate metabolism and oxygen consumption is so high, in contrast to epinephrine, that these last two functions must be regarded as specific for epinephrine.

The Biosynthesis of Norepinephrine

A quick survey of the state of knowledge on the biosynthesis of norepinephrine leads to the following three metabolic possibilities:



Belief that the route from 3,4-dihydroxyphenylalanine (dopa) through dopamine to norepinephrine is the actual biosynthetic pathway has been confirmed by several observations. There is a highly specific L-dopadecarboxylase in several mammalian tissues. The enzyme was first discovered in kidney by Holtz, Heise and Lüdtke (10) in 1938. Three years later, Holtz, Credner and Koepp (11) reported the presence of dopamine in urine. The subsequent detection of 3,4-dihydroxyphenylalanine and dopamine in normal mammalian heart and in suprarenal extracts from thyroidectomized sheep by Goodall (12, 13) suggested to him that dopamine might be an intermediate in the synthesis of norepinephrine and that thyroxine might play a role in this synthesis. The detection of the same compounds in the adrenal gland of the ox by Langemann (14) lent further support to this idea.

Blaschko et al. (15) reported that rats fed on a pyridoxine deficient diet and killed eighteen hours after a dose of insulin, had greatly reduced amounts of epinephrine in their adrenals, as compared to rats on a normal diet treated in the same manner. This was attributed to a failure of resynthesis of the hormone after depletion by insulin; it is known that pyridoxine is converted to pyridoxal phosphate, a necessary cofactor of decarboxylases. Von Euler and Uddén (16) demonstrated in 1952 the presence of increased amounts of norepinephrine in several organs of the cat after administration of 3,4-dihydroxyphenylalanine, tyrosine and other hydroxyphenyl compounds. As early as 1947, Gurin and Delluva (17) reported that radioactive epinephrine could be found in rat adrenals after they had ingested phenylalanine labelled with C^{14} and with tritium. More recently, the pathway from phenylalanine to the catechol amines has been confirmed

by several groups of workers. Demis, Blaschko and Welch (18) in 1955 detected labelled dopamine and norepinephrine in bovine adrenal homogenates which had been incubated with 3,4-dihydroxyphenylalanine-2-C^{14}. One year later, Udenfriend and Wyngaarden (19) injected C^{14}-labelled compounds into rats and showed that phenylalanine, tyrosine and 3,4-dihydroxyphenylalanine are precursors of norepinephrine and epinephrine and that phenylethylamine and tyramine are not. This was confirmed by Goodall and Kirshner (20) in 1957, who showed by incubating adrenal slices with labelled compounds that tyrosine and 3,4-dihydroxyphenylalanine are converted to dopamine, norepinephrine and epinephrine, that dopamine is converted to norepinephrine and epinephrine, and that tyramine is not.

When no definite evidence yet existed for the introduction of an hydroxyl group into the side chain of dopamine, some workers believed that the hydroxylation might occur before the decarboxylation. They tested dihydroxyphenylserine as a substrate for decarboxylases. In 1950, Beyer (21) reported the decarboxylation of the compound by mammalian enzyme preprations. In an analogous fashion, L-serine has been shown to be converted to ethanolamine in the rat (22). Schmiterlöw (23) demonstrated with rabbits that the norepinephrine content of urine is markedly increased after the injection of small amounts of dihydroxyphenylserine. Although no further work has been done on this pathway, these results certainly illustrate its possible occurrence.

A third route to norepinephrine, that via p-hydroxyphenylethanolamine, is still not well established. p-Hydroxyphenyl and m-hydroxyphenyl radicals can be oxidized under the influence of ultraviolet radiation to the

dihydroxy radical; this was accomplished by Erspamer (24, 25) with octopamine. This work led to the idea that a monohydroxyphenylserine might serve as a precursor of norepinephrine. In fact, Werle and Peschel (26) found that extracts of guinea-pig kidney and of various sheep organs slowly decarboxylate and oxidize p-hydroxyphenylserine to yield octopamine. Octopamine, however, has been found to date only in the salivary glands of Octopus vulgaris (25). For this reason, ring oxidation of a phenylethanolamine as a possible last step in the biosynthesis of norepinephrine remains uncertain.

The Metabolism of Norepinephrine

The metabolic fate of norepinephrine is still rather poorly elucidated. Norepinephrine has been detected in urine. Von Euler and Lüft (27) found that only 6% of infused norepinephrine appeared in urine. Urinary elimination of the endogenous compound by healthy subjects has been established at 10 to 60 micrograms per 24 hours (28). The direct excretion of norepinephrine therefore does not appear to play an important role in its disappearance from the organism. It should be mentioned however, that a urinary excretion of 3000 micrograms of norepinephrine in 24 hours has been recorded for a patient with a pheochromocytoma, a tumor of the chromaffin cells (29).

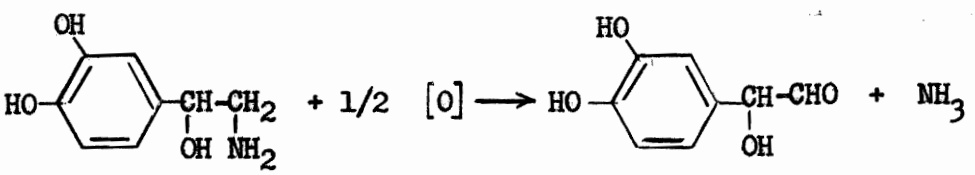
The transformation of norepinephrine into its homologue, epinephrine, is certainly an important avenue in the metabolism of the compound. Bülbring (30), in 1949, demonstrated that adrenal tissue methylates norepinephrine and that the process requires adenosine triphosphate.

Lockett (31), using adrenal tissue from several species, confirmed this observation; she showed also that demethylation of epinephrine occurs in many cases. Adrenal homogenate experiments by Goodall and Kirshner (20) provided more evidence for the methylation of norepinephrine to epinephrine. Finally, Matsuoka et al. (32) demonstrated last year that C^{14}-norepinephrine is converted to epinephrine in vivo.

Several good reviews on the enzymic inactivation of norepinephrine are available (33-36). For this reason, only a brief summary of the literature in this field is presented here.

Amine oxidase, one of the enzymes which destroy norepinephrine, is widely distributed in animal tissues. Its presence has been demonstrated in many tissues where the catechol amines are found; it exists in all vertebrates and all these animals contain chromaffin tissue. Significantly in the earthworm amine oxidase has been found only in the gut and the gut is the only tissue of this animal supplied by adrenergic nerves (36).

Amine oxidase catalyzes the oxidative deamination of norepinephrine:



norepinephrine

3,4-dihydroxy-mandelaldehyde

The aldehyde is then oxidized to dihydroxymandelic acid by aldehyde oxidase. These reactions were first observed in 1928 by Hare (37) and were confirmed by Blaschko, Richter and Schlossmann (38) eleven years

later. Schayer et al. (39), in 1954, studied the effect of inhibitors of amine oxidase on the metabolism of α-C^{14}-tyramine in vitro; they found that Marsilid and choline p-tolyl ether inhibited the degradation of tyramine, while others, like benzedrine and ephedrine did not. Schayer concluded that the effect of the inhibitors could hardly be due to an action on amine oxidase. Von Euler (40) pointed out, however, that even a moderate inhibition of the amine oxidase activity at the site of transmitter release and inactivation might influence the adrenergic nerve transmitter to a higher degree than would be the case for exogenously administered amines. Schayer et al. (41) later injected radioactive norepinephrine into rats and demonstrated the existence of three major metabolic products in the urine by paper chromatography; the formation of one of the compounds was prevented by pretreatment of the animals with Marsilid or with other amine oxidase inhibitors. Thus it may be concluded that amine oxidase might play an important role in the inactivation of norepinephrine.

Imaizumi and Kawamoto (42, 43), in 1952, obtained an apoenzyme preparation from blood which appeared to convert epinephrine to its keto derivative, adrenalone, in the presence of diphosphopyridine nucleotide and flavineadeninedinucleotide. Norepinephrine might also be a substrate for this apoenzyme.

One of the early products of oxidation of norepinephrine in vitro is noradrenochrome which then gives rise to a dark melanin-like compound. The reactions happen spontaneously in neutral or weakly alkaline solutions. Noradrenochrome was first identified by Beaudet (44) in 1951.

Norepinephrine however, has been found to be very stable in blood and plasma (45). Furthermore, the chromatographic studies by Schayer and Smiley (41) appear to indicate that noradrenochrome is probably not an important intermediate in the metabolism of norepinephrine. On the other hand, the peroxidase and cytochrome oxidase systems in cells may account for a considerable part of norepinephrine inactivation. Philpot and Cantoni (46) have reported that after selective inhibition of the amine oxidase of the liver with methylene blue, the liver preparation still retained 20 to 30% of its inactivating power; in contrast, in heart and skeletal muscle, the major part of the inactivation of infused epinephrine was lost after treatment with cyanide. Lund (47), in 1951, measured the rate of disappearance from blood of injected norepinephrine; he found that the largest part of a dose was eliminated within three minutes and that no trace of norepinephrine could be detected after eight minutes. He assumed that this disappearance takes place by diffusion into the tissues and that liver amine oxidase and muscle cytochrome oxidase are responsible for the metabolism. Wajzer (48) supports this view. The inactivation noted in these experiments could not be due to destruction of norepinephrine in the blood because of the presence of protective substances in that fluid. It is possible then that, in cells, norepinephrine may be oxidized to noradrenochrome by the action of cytochrome oxidase.

In summary, it may be stated that urinary excretion plays a relatively unimportant role in the inactivation of norepinephrine; the contribution of epinephrine dehydrogenase is not yet established. The cytochrome oxidase system may account for a large part of the inactiva-

tion in the cells. Finally, there is much evidence to suggest that a significant amount of norepinephrine is destroyed by amine oxidase.

In the present study, the elucidation of the fate of norepinephrine after oxidative deamination is attempted. The project was initiated by an observation made by Armstrong et al. (49) in their systematic survey of the phenolic acids in urine. One of the compounds observed on their chromatograms, number 10 (49), appeared to be an endogenous metabolite of an aromatic amino acid - the amount of this metabolite in urine did not seem to be affected by diet. The chromatographic behavior of this compound suggested that it might be 3-methoxy-4-hydroxymandelic acid. Logical biological precursors of a compound of this type might very well be the catechol amines; the action of amine oxidase and aldehyde oxidase to produce 3,4-dihydroxymandelic acid from norepinephrine has been mentioned. The remaining transformation would consist then in the methylation of a phenolic hydroxyl group. Shaw, McMillan and Armstrong (50) have demonstrated that this type of reaction takes place in the transformation of homoprotocatechuic acid to homovanillic acid during the course of the metabolism of 3,4-dihydroxyphenylalanine.

This thesis describes the synthesis of the organic compounds implicated in this pathway and the metabolic experiments which were carried out to establish the biological reactions. The significance of the results obtained is also discussed.

PREPARATION OF ORGANIC COMPOUNDS

Both epinephrine and norepinephrine are substrates for amine oxidase (35); under the influence of this enzyme, they are presumably converted to the aldehyde which then undergoes oxidation to the corresponding dihydroxymandelic acid. Dihydroxymandelic acid might be eliminated unchanged by the organism or might undergo further reactions. A likely possibility, mentioned at the end of the preceding section, could be the methylation of one of the phenolic hydroxyl groups. However, only indirect evidence has been advanced to support the role of dihydroxymandelic acid in the metabolism of norepinephrine and its homologue: these compounds lose ammonia and methylamine, respectively, when incubated with tissue containing amine oxidase (38).

To test these hypotheses by metabolic experiments, 3-methoxy-4-hydroxymandelic acid and 3,4-dihydroxymandelic acid were synthesized from the appropriate aldehydes. The methoxyhydroxymandelic acid was resolved by means of its cinchonine salts and the configuration of its enantiomorphs was assigned following resolution of the racemic amide with stereospecific L-leucine aminopeptidase. The enzymic resolution is included in this section, inasmuch as it confirms the results of the chemical approach. Improvements in the yield and the purity of the compounds synthesized will be discussed in the light of the historical background of these compounds and of the methods employed.

3-Methoxy-4-hydroxymandelic Acid and Related Compounds

3-Methoxy-4-hydroxy-DL-mandelic acid was prepared in 45% overall yield from vanillin, via the cyanohydrin and the ester; Gardner and Hibbert (51) have reported a yield of 19% by this route. A new approach to this compound, involving reaction of guaiacol with sodium glyoxylate, is described in a recent patent (52).

3-Methoxy-4-hydroxymandelonitrile was obtained from the aldehyde and liquid hydrogen cyanide by Makarov and Gorskii (53); they reported difficulty in purifying the product. Buck (54) prepared the compound in 56% yield by the reaction of the aldehyde with 2 moles of sodium bisulfite and 4 moles of potassium cyanide. Hahn et al. (55), using approximately half as much bisulfite and cyanide, reported an 89% yield of a nitrile of uncertain purity, obtained directly by evaporation of extracts. The product obtained by still others (51, 56) with these procedures has been used without isolation, because of reputed instability (51). In the present study, the nitrile was obtained in 80% yield by use of 4 moles of bisulfite and 4 moles of cyanide; a yield of 25 to 35% of a less pure product was achieved with the use of 1.1 to 1.2 moles of bisulfite and cyanide. Crystalline 3-methoxy-4-hydroxymandelonitrile is stable for many months at 5° and shows no signs of deterioration during shorter periods at room temperature.

The nitrile has been converted, without isolation, to ethyl 3-methoxy-4-hydroxymandelate by the action of dry hydrogen chloride and ethanol, via the iminoester hydrochloride, in a yield of 25%, based on the aldehyde (51, 56); crystalline nitrile was reported to yield only 3% of ester (56). The ester was then saponified to the acid in 80% yield (51). Perhaps the

solid nitrile used in earlier work (56) contained vanillin or other impurities - a 40% yield of ester has been obtained from pure nitrile in the present work whereas the impure compound produced lower yields of impure ester. A more complete recovery of the ester was impeded by the presence of byproducts. A better yield of acid, 56% based on nitrile, was obtained by saponifying the intermediate iminoester hydrochloride without isolating the ester. 3-Methoxy-4-hydroxymandelic acid has been reported to resinify on exposure to air (51); in this work, the ester and the acid have proved to be stable indefinitely.

3-Methoxy-4-hydroxy-DL-mandelamide has been prepared by Schwartz and McCarthy (56) by allowing the ester to stand for 11 days in an ammonia-saturated ethanol solution; they reported a 65% yield of crude amide (no melting point is cited) which was recrystallized four times to give a product melting at 136.5-137.5°. In this study, a yield of 75% of crude amide was obtained by the same procedure, m.p. 129-130°. This was considered to be sufficiently pure for enzyme treatment.

3,4-Dihydroxymandelic Acid and Related Compounds

3,4-Dihydroxymandelic acid was synthesized in 23% over-all yield from protocatechualdehyde, via the cyanohydrin and the ester. Relatively high purity of the intermediates used in each step of the synthesis was found to be essential. The only previous preparation of dihydroxymandelic acid (57) started with piperonal and yielded, by way of an involved procedure, a highly unstable and very impure product.

Condensation of protocatechualdehyde with sodium bisulfite and potassium cyanide, or with liquid hydrogen cyanide, is claimed in an early German patent (58) to form impure 3,4-dihydroxymandelonitrile. Buck (54) used this method to obtain the nitrile in 63% yield. In the present study, a similar yield was obtained with hydrogen cyanide but the product was impure and the procedure hazardous. Nitrile of good purity was prepared in 64% yield by Shaw et al. who used 4 moles of bisulfite and 4 moles of cyanide (59). Lower proportions of bisulfite and/or cyanide, as well as addition of hydrochloric acid to the condensation mixture to maintain the pH at 7.0 ± 0.2, resulted in lower yields of product. The stability of the crystalline cyanohydrin was found to be similar to that of its methylated analogue.

3,4-Dihydroxymandelonitrile was converted to the ester in 50% yield by the action of dry hydrogen chloride and ethanol; the yield was lowered when insufficient time was allowed for the formation of the intermediate iminoester hydrochloride. Dihydroxymandelic acid was obtained in 85% yield by the saponification of the ester under nitrogen. The sensitivity of the acid to atmospheric oxidation (in alkaline solution) made it expedient to isolate and purify the ester, thus avoiding prolonged handling of the acid. The stability of dihydroxymandelic acid and of its ester was found to be comparable to that of their methylated analogues.

3,4-Dibenzyloxy Compounds

The synthesis of dihydroxymandelic acid also was attempted by the use of dibenzyloxybenzaldehyde as an intermediate. This compound was prepared in quantitative yield from protocatechualdehyde by the action of benzyl chloride in the presence of anhydrous potassium carbonate. This synthesis has been reported previously (60, 61). Several attempts to convert this material to the corresponding nitrile failed to yield a product which could be isolated in significant amounts. The relative insolubility of the dibenzyloxybenzaldehyde in a medium of sufficient polarity to allow intimate contact with the cyanide probably led to these results. Some bisulfite addition compound was obtained however. Small amounts of impure nitrile were prepared with the use of liquid hydrogen cyanide. The synthesis of the nitrile has been reported subsequently by Bristow (61) who refluxed a mixture of aldehyde, potassium cyanide, glacial acetic acid and absolute alcohol, according to a method developed by Butenandt and Schmidt-Thomé (62) in their work on dehydroandrosterone; no physical constants were given for the nitrile. Satisfactory progress in the conversion of protocatechualdehyde to dihydroxymandelic acid made it expedient to abandon the dibenzyloxy route.

Resolution of 3-Methoxy-4-hydroxy-DL-mandelic Acid

The resolution of the racemic synthetic product was attempted with an assortment of alkaloids and solvents. Quinine was first tried because it has been used successfully with unsubstituted mandelic acid (63); this

route was soon abandoned however, because the quinine salts of 3-methoxy-4-hydroxymandelic acid underwent appreciable hydrolysis during recrystallization from water. Small runs using all possible combinations of brucine, cinchonine and strychnine with water, methanol, ethanol, isopropyl alcohol and ethyl acetate were then made for preliminary observations. The acid was finally resolved by the cinchonine - water system in quantities sufficient for biological experiments; the optical rotation of the enantiomorphs was established at +133° and -133° ($[\alpha]_D^{22}$; c = 1, absolute ethanol). A small amount of pure cinchonine 3-methoxy-4-hydroxy-D-mandelate was prepared by several recrystallizations from ethanol to yield a product which did not change in properties after further recrystallizations; the salt decomposed at 204° and had a rotation of $[\alpha]_D^{24}$ + 89° (c = 1, water).

3-Methoxy-4-hydroxy-DL-mandelamide was submitted to the action of L-leucine aminopeptidase, an enzyme of high stereospecificity. The enzymic hydrolysis was graciously carried out by Hill, using a method which he reported earlier (64). A continuous extraction process was employed to separate the liberated acid from the unhydrolyzed amide. The D-mandelamide melted at 162-163° and had a rotation of $[\alpha]_D^{20}$ -81° (c = 1, water). The acid obtained by the procedure decomposed at 151° and had a rotation of $[\alpha]_D^{22}$ +128° (c = 1, water). Since leucine aminopeptidase hydrolyzes compounds with the L-configuration only, according to Smith and Spackman (65), and since the enantiomorph liberated from the DL-amide by leucine aminopeptidase has a positive optical rotation, the isomers of the acid can be properly referred to as 3-methoxy-4-hydroxy-D(-) and L(+) mandelic acid.

Experimental Procedures

Before describing the chemical procedures employed in the synthetic work, it should be mentioned that the technique of paper chromatography was employed widely and was found to be quite useful in following the course of various reactions and in estimating the composition of reaction products. Shaw (59) has made a systematic survey of the chromatographic behavior of the mandelic derivatives prepared here; his data are included in table I, on page 31.

3-Methoxy-4-hydroxymandelonitrile. 152.4 g. (1.0 mole) of vanillin (U.S.P. grade) was dissolved in a solution of 416.3 g. (4.0 moles) of sodium bisulfite and 875 ml. of water. To this mixture was added dropwise, during 30 minutes, a solution of 260.4 g. (4.0 moles) of potassium cyanide in 400 ml. of water. The reaction mixture was stirred vigorously and the temperature was kept between -10° and +12° during the addition and for 30 minutes thereafter. Enough water was added to dissolve the white crystals which precipitated during the reaction. The clear solution was extracted with four 600-ml. portions of ether. The combined ether extracts were washed with two 100-ml. portions of 4 N aqueous sodium bisulfite solution, dried over anhydrous sodium sulfate, treated with Norite and evaporated *in vacuo* under nitrogen to yield a white crystalline residue. The solid residue was dissolved in 200 ml. of anhydrous ether and 400 ml. of benzene was added to the solution. After refrigeration at 5°, 124.0 g. (69% yield) of the nitrile was recovered as colorless crystals, m.p. 81-83° [Lit. (54) m.p. 83°]. The mother liquor was concentrated *in vacuo* to a yellow oil

which, after recrystallization from 1:2 ether - benzene, gave another 19.5 g. (11% yield) of the product, m.p. 82-84°. The melting point of a mixture of the two crops was not depressed but a mixture of either crop with vanillin (m.p. 82°) melted below 65°. The nitrile retained solvent tenaciously; prior to prolonged air drying, freshly isolated compound melted over a wide temperature range. Further crops from the mother liquor contained increasing amounts of vanillin.

Ethyl 3-Methoxy-4-hydroxymandelate. A solution of 89.6 g. (0.50 mole) of the nitrile in a mixture of 24.0 g. (0.52 mole) of absolute ethanol and 400 ml. of anhydrous ether was treated at 5° with dry hydrogen chloride until 19.3 g. (0.53 mole) of the gas was absorbed (15 minutes). Care was taken to exclude atmospheric moisture from the reaction mixture. During storage at 5° for 48 hours, gummy crystals of the iminoester hydrochloride separated. The ether supernatant was discarded and the gummy residue was thoroughly dried *in vacuo* over potassium hydroxide to remove excess hydrogen chloride and traces of ether. The dry iminoester hydrochloride was stirred with 900 ml. of water for four hours at room temperature; 10 ml. of oil which separated was shaken with three 100-ml. portions of water. All aqueous layers were combined and treated with Norite; the filtrate was used either for isolation of the ester or for direct preparation of the acid.

For the isolation of the ester, the aqueous solution was extracted with four 750-ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate, treated with Norite and evaporated

in vacuo under nitrogen to a yellow oil. The oil was dissolved in 750 ml. of ether and 900 ml. of cyclohexane was added to the solution. After 24 hours at 5°, the resulting crystal cake was fragmented and 225 ml. more of cyclohexane was added. Another day at 5° gave, after isolation, 45.1 g. (40% yield) of the ester, m.p. 74-76°. A sample recrystallized from water, with Norite treatment, was recovered in the form of colorless blunt needles, m.p. 77-78° [Lit. (51) m.p. 77°], unchanged by further recrystallization from ether -cyclohexane. The ether - cyclohexane mother liquor was concentrated in vacuo to yield 34.5 g. of yellow oil which failed to give more crystalline ester.

The oil was hydrolyzed with 1 N aqueous sodium hydroxide, in the manner described in the next preparation, to give 11.5 g. (12% yield, based on nitrile) of 3-methoxy-4-hydroxymandelic acid, dec. 131-133°.

3-Methoxy-4-hydroxy-DL-mandelic Acid. 150 ml. of 10 N aqueous sodium hydroxide was added to an aqueous hydrolysate of the iminoester hydrochloride (prepared in the manner described in the preceding section) and the resulting basic solution was refluxed in a nitrogen atmosphere for two hours. The solution was cooled to room temperature, adjusted to pH 7.0 with concentrated hydrochloric acid and extracted with six 900-ml. portions of ethyl acetate to remove neutral impurities. The aqueous phase was acidified to pH 1.5 and was extracted again with six 900-ml. portions of ethyl acetate. The latter extracts were combined, dried over anhydrous sodium sulfate, treated with Norite and then concentrated in vacuo to dryness. The hard brown residue was dissolved in 600 ml. of boiling ethyl acetate, the solution was treated with Norite, and 900 ml. of cyclohexane was added to the filtrate.

After refrigeration at 5°, 52.2 g. (53% yield, based on nitrile) of the methoxyhydroxymandelic acid was obtained, dec. 130-132° (sample in bath at 125°; heating rate: 2° per minute). Three grams (3% yield) more of acid was obtained from the mother liquor, dec. 129-131°. The compound was recrystallized from boiling acetonitrile with Norite treatment; 80% of the acid was recovered as a hard faintly yellow crystalline scale, dec. 131-133° [Lit. (51) m.p. 133°].

25.3 g. (0.11 mole) of ester, m.p. 74-76°, in 225 ml. of 1 N sodium hydroxide was processed in the manner just described to yield two crops of acid from the ethyl acetate - cyclohexane recrystallization system: 17.0 g. (77% recovery), dec. 131-133°, and 1.2 g. (5%), dec. 130-132°.

3,4-Dihydroxymandelonitrile. A solution of 13.0 g. (0.26 mole) of sodium cyanide was added dropwise during 30 minutes to a mixture of 150 ml. of ethyl acetate with 34.5 g. (0.25 mole) of protocatechualdehyde and 27.2 g. (0.26 mole) of sodium bisulfite dissolved in 250 ml. of water. The reaction mixture was stirred vigorously and maintained at temperatures below -5° during the addition and for 30 minutes thereafter. The ethyl acetate layer was separated; the aqueous phase was saturated with sodium chloride and was extracted with five 150-ml. portions of ethyl acetate. The combined ethyl acetate layers were washed with two 25-ml. portions of 4 N sodium bisulfite solution, dried over anhydrous sodium sulfate, treated with Norite and concentrated to dryness in vacuo. The oily residue (32.2 g.) was dissolved in 64 ml. of ether; an equal volume of 1,2-dichloroethane and two volumes of petroleum ether (b.p. 30-60°) were added successively; this led to the separation of a dark red oil.

During two days at 5°, the oil hardened to a light brown mass and glistening crystals appeared in the supernatant. These materials were separated from each other by decantation and filtration; 10.3 g. (25% yield) of the white crystals was obtained, m.p. 97-98°. The filtrate was concentrated to dryness in vacuo under nitrogen. The residue was combined with the hardened oil (m.p. 96-99°) and was dissolved in 200 ml. of 1:1 ether - dichloroethane mixture; enough petroleum ether was added to the solution to obtain incipient turbidity and the resulting mixture was stored at 5°. During the next two days, more petroleum ether was added in 50 ml. increments (to a total solution volume of 900 ml.) until the crystallization process was completed; 12.7 g. of light brown crystalline powder was isolated. Recrystallization of this second crop from the 1:1:7 ether -dichloroethane - petroleum ether mixture by the same technique produced 11.8 g. (28% yield) of hard cream-colored rosettes, m.p. 97.5-99° [Lit. (54) m.p. 95°]. A total of 22.0 g. (53% yield) of pure nitrile was thus obtained by the procedure; further processing of the mother liquor was not attempted.

Ethyl 3,4-Dihydroxymandelate. Dry hydrogen chloride was passed into a solution of 11.77 g. (0.071 mole) of nitrile in a mixture of 3.37 g. (0.073 mole) of absolute ethanol and 50 ml. of anhydrous ether at 5°, until 2.67 g. (0.073 mole) of the gas was absorbed (15 minutes). The mixture was stored at 5° for 72 hours, during which time gummy crystals of the iminoester hydrochloride separated. The ether supernatant was discarded and the gummy residue was dried in vacuo over potassium hydroxide. The solid was dissolved in 90 ml. of water and the solution was allowed to stand one hour at

room temperature, was saturated with sodium chloride, and the aqueous solution was shaken with five 90-ml. portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, treated with Norite and evaporated *in vacuo* to yield 12.9 g. of gummy cream colored powder. The powder was dissolved in 65 ml. of absolute ethanol and an equal volume of cyclohexane was added to the solution. After 24 hours at 5°, another volume of cyclohexane was added and the solution was returned to the refrigerator for another day; 7.16 g. (51% yield) of ester were recovered in two crops, m.p. 152-154° [Lit. (54) m.p. 152-153°].

3,4-Dihydroxymandelic Acid. A solution of 5.0 g. (0.024 mole) of ester in 100 ml. of 2 *N* sodium hydroxide was stirred for four hours under nitrogen at room temperature. The solution was acidified to pH 1.0 with concentrated hydrochloric acid, saturated with sodium chloride and extracted with five 150-ml. portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, treated with Norite and evaporated *in vacuo* to yield 4.0 g. of white powder. The powder was recrystallized from 200 ml. of 1:1 ethyl acetate - cyclohexane mixture; 3.67 g. (85% yield) of hard pale yellow rosettes was obtained, dec. 136.5° (sample in bath at 134°; heating rate: 2° per minute). Another recrystallization of this dihydroxymandelic acid from the same solvent mixture with charcoal treatment produced colorless needles (91% recovery), dec. 137°.

Analysis (66). Calculated for $C_8H_8O_5$: C, 52.18; H, 4.38. Found: C, 51.46; H, 4.35. [Lit.:the product of Barger and Ewins (57) was a white amorphous powder which could not be crystallized. No melting point was cited.

Analysis. Calculated for $C_8H_8O_5$: C, 52.18; H, 4.38. Found: C, 53.0; H, 5.9; these values were corrected by the authors by subtracting from the weight of the sample an unrecorded weight of ash which remained after combustion.]

Separation of the Cinchonine 3-Methoxy-4-hydroxy-D and L-mandelates. A pulverized mixture of 45.0 g. (0.153 mole) of cinchonine (Merck, U.S.P.) and 30.4 g. (0.153 mole) of the DL-acid was simmered with 550 ml. of water; 5 g. of undissolved alkaloid was removed by filtration and was washed with two 25-ml. portions of boiling water. The combined filtrate and washings were seeded with some cinchonine salt of the leverotatory acid which had been obtained previously from a small test batch. The solution was allowed to cool slowly in a 65° water bath. At 60°, a small amount of brown oil separated; it failed to redissolve on addition of 100 ml. of water and heating to boiling. The boiling solution was treated with 6.0 g. of Norite and was then filtered; the charcoal cake was washed with two 20 ml. portions of boiling water and these washings were added to the filtrate. The pale yellow filtrate was seeded again and was allowed to cool slowly to 40° in a water bath. It was then stored at 37° for 12 hours and at room temperature for 24 hours. The crystalline salt was collected on a filter, washed with small volumes of cold water, cold ethanol and ether. The crop was dried in vacuo over phosphorus pentoxide; 31.8 g. (42.1% yield) of cream-colored crystals was recovered, dec. 196° (sample in bath at 190°; heating rate: 3° per minute), $[\alpha]_D^{23}$ + 106° (c = 1, absolute ethanol). This salt was used directly for the preparation of the D-acid,

and the mother liquor for the L-acid.

Isolation of 3-Methoxy-4-hydroxy-(-)-mandelic Acid from its Crude Cinchonine Salt. A suspension of 31.7 g. (0.065 mole) of the dextrorotatory cinchonine salt in 140 ml. of 0.5 N sodium hydroxide was stirred for 30 minutes and filtered; the cinchonine cake was washed with four 20-ml. portions of water. The combined filtrate and washings were extracted with three 50-ml. portions of chloroform to remove traces of cinchonine. The aqueous phase was acidified to pH 0.5 with hydrochloric acid, saturated with sodium chloride and extracted with four 100-ml. portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and concentrated in vacuo to yield 7.5 g. of gummy crystals. Another 5.6 g. of the mandelic acid was obtained by repeating the above procedure with the residual cinchonine. The 12.7 g. (100% recovery) of crude acid was dissolved in 60 ml. of boiling acetonitrile, treated with Norite, filtered, diluted to 90 ml. with the charcoal washings and stored at room temperature. The compound was obtained as a crystalline scale (7.1 g.), $[\alpha]_D^{22}$ -61° (c = 1, water). This material was recrystallized wastefully from 100 ml. of acetonitrile to yield 2.0 g. of rhomboids, dec. 151° (sample in bath at 145°; heating rate: 3° per minute), $[\alpha]_D^{23}$ -124° (c = 1, water). The mother liquor from this crop was reworked to yield 2.6 g. of hexagonal plates of the DL-acid, dec. 132-133°, $[\alpha]_D^{23}$ 0° (c = 1, water), and 0.8 g. of crude levoratatory acid, dec. 137-138°, $[\alpha]_D^{22}$ -72° (c = 1, water). These last two crops were not processed further. A final recrystallization of 1.93 g. of the rhomboids from 20 ml. of acetonitrile yielded 1.57 g. of levorotatory 3-methoxy-4-hydroxy-

mandelic acid, dec. 150-151.5°, $[\alpha]_D^{22}$ -133° (c = 1, water). Further recrystallization did not change the properties of this compound.

In another run, crystalline cinchonine salt from 10 g. of racemic acid was subjected to four successive wasteful recrystallizations from absolute ethanol; 1.6 g. of the cinchonine salt of the levorotatory acid was obtained, dec. 204°, $[\alpha]_D^{24}$ +89° (c = 1, absolute ethanol); these properties were not changed by further recrystallization. The levorotatory acid was regenerated from this pure salt in the manner described above, dec. 152°, $[\alpha]_D^{24}$ -131° (c = 1, water).

Isolation of 3-Methoxy-4-hydroxy-(+)-mandelic Acid from the Cinchonine Salt Filtrate. The mother liquor from the crude dextrorotatory cinchonine salt was treated with sodium hydroxide and extracted in the manner described for the crystalline dextrorotatory salt to yield 16.8 g. (100% recovery) of crude acid. This was crystallized from 150 ml. of acetonitrile to give 4.4 g. of a crystalline scale, $[\alpha]_D^{22}$ +66° (c = 1, water). This material was recrystallized wastefully from 100 ml. of acetonitrile to yield a first crop of 0.9 g. of rhomboids, dec. 152°, $[\alpha]_D^{22}$ +135° (c = 1, water) and a second crop of 1.2 g., dec. 151°, $[\alpha]_D^{22}$ +126° (c = 1, water). These two crops were combined and recrystallized from 22 ml. of acetonitrile to yield 1.72 g. of dextrorotatory 3-methoxy-4-hydroxymandelic acid, dec. 151°, $[\alpha]_D^{22}$ +133° (c = 1, water).

3-Methoxy-4-hydroxy-DL-mandelamide. 1.45 g. (0.006 mole) of ethyl 3-methoxy-4-hydroxymandelate was dissolved in 75 ml. of methanol previously saturated at 0° with anhydrous ammonia. The solution was allowed to stand at room

temperature for 48 hours in a pressure bottle. The alcohol and excess ammonia were removed by evaporation in vacuo to give 1.33 g. of red gum. The residue was dissolved in 10 ml. of boiling n-propyl alcohol and the solution was treated with Norite: no decolorization was observed. The filtrate was re-heated to boiling and diluted with boiling cyclohexane to the point of incipient turbidity. After storage at room temperature for 24 hours, 1.0 g. (83% yield) of cream-colored powder was obtained, m.p. 125-128°. This product was very soluble in alcohols, less soluble in hot ethyl acetate and hot acetonitrile, and slightly soluble in benzene, ether and chlorinated solvents.

The crude amide was recrystallized from boiling ethyl acetate with Norite treatment to afford 0.77 g. (63% yield) of fine white crystals, m.p. 129-130° (heating rate: one degree per minute near melting point). [Lit. (56) m.p. 136.5-137.5°]. This material was considered to be sufficiently pure for enzyme treatment.

Analysis. (66). Calculated for $C_9H_{11}O_4N$: N, 7.10. Found: N, 7.47.

Hydrolysis of 3-Methoxy-4-hydroxy-L-mandelamide by Leucine Aminopeptidase. Five milliliters of a solution of leucine aminopeptidase (C_1 = 30)* containing 2.9 mg. of protein per milliliter was pipetted into a 25 ml. volumetric flask with 3 ml. of 0.05 M pH 8.5 tris-(hydroxymethyl) aminomethane (Tris buffer) and 0.5 ml. of 0.025 M manganous chloride. The enzyme was activated

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* (C_1) is a gross index of purity. It corresponds specifically to the first order rate constant for hydrolysis of L-leucinamide and is expressed in decimal logarithms per milligram of enzyme nitrogen.

by incubating this mixture at 40° for 30 minutes. The following ingredients were then added to the activated enzyme: a 0.25 M solution of 500 ml. of DL-amide in 10 ml. of deionized water and adjusted to pH 8.5 with 1 N sodium hydroxide; 2 ml. of 0.025 M manganous chloride and 5 ml. of 0.05 M pH 8.5 Tris buffer. The volume of the reaction mixture was adjusted to 25 ml. with deionized water. The resulting solution was kept at 40° for 44 hours. After 25 hours of incubation, 95% of the L-amide was hydrolyzed, as determined by microtitration of the liberated ammonia by the method of Grassmann and Heide (67).

Isolation of the Products of Enzymic Hydrolysis. The solution prepared by enzymic digestion was diluted to 100 ml. with water and was adjusted to pH 7.0 with concentrated hydrochloric acid. The unhydrolyzed D-amide was isolated from this mixture by extracting it with boiling ethyl acetate in a continuous extractor for 18 hours. The process was run at a subdued rate because of a tendency toward emulsification which was probably caused by the trace of enzyme in the aqueous phase. The two phases were separated. An estimate of the distribution of the liberated acid and of the amide between the two solvents was obtained by means of paper chromatography in an isopropyl alcohol - ammonium hydroxide - water system (8:1:1), with color development by diazotized sulfanilic acid and quantitation of the compounds by comparison with reference solutions of the acid and of the amide. This method of estimation will be described later, in the biochemical section of this work. The ethyl acetate layer contained approximately 252 mg. of amide and no detectable quantity of acid; in contrast,

20 mg. of amide and 215 mg. of acid were found in the aqueous phase.

The aqueous phase was acidified to pH 1.2 with concentrated hydrochloric acid, saturated with sodium chloride and extracted four times with equal volume portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield a greenish oil. The oil was dissolved in 3.5 ml. of boiling acetonitrile and the solution was refrigerated at 5° for 24 hours to afford 88 mg. of crude crystalline acid. Concentration of the mother liquor and recrystallization of the residue from acetonitrile yielded additional crops of 64 and 25 mg. of crude acid. The crude product, 177 mg. in all, was recrystallized from an acetonitrile solution with Norite treatment; 125 mg. (71% recovery) of small hexagonal rods was obtained, dec. 151° (sample in bath at 149°; heating rate: 2° per minute); $[\alpha]_D^{22}$ +128° (*c* = 1, water). Further treatment of the mother liquor in the same manner gave an additional 31 mg. (17%) of like material, dec. 148°, $[\alpha]_D^{20}$ +115° (*c* = 1, water). A total of 156 mg. or 63% of the original 3-methoxy-4-hydroxy-L(+)-mandelic acid from the DL-amide was thus isolated in purified form.

The ethyl acetate solution obtained by continuous extraction of the enzyme reaction mixture was dried over anhydrous sodium sulfate and evaporated *in vacuo* to yield a thin film of crystals and gum. This residue was dissolved in 50 ml. of boiling ethyl acetate and the solution was stored at 5°. Filtration of the deposited material yielded 155 mg. of white crystalline powder. Concentration of the mother liquor and crystallization of the residue from the same solvent yielded an additional crop

of 36 mg. of similar material. These crops were combined and redissolved in a minimum of boiling absolute ethanol containing 1 ml. of ethyl acetate (to avoid large excess of solvent). The hot saturated solution was treated with Norite and stored at 5°. After complete crystallization, 93 mg. (49% recovery) of fine white needles was isolated. The mother liquor was reworked in the same manner to produce 51 mg. (27%) more of the amide. The two crops were combined prior to elemental analysis and measurement of physical constants. 144 mg. or 58% of the original 3-methoxy-4-hydroxy-D-mandelamide was thus recovered from the racemic mixture, m.p. 162-163°, $[\alpha]_D^{20}$ -81° (c = 0.8, water).

Analysis (66). Calculated for $C_9H_{11}O_4N$: C, 54.81; H, 5.62; N, 7.10. Found: C, 54.77; H, 5.73; N, 7.07.

TABLE I

Chromatographic Behavior

of Selected Mandelic Derivatives and Phenolic Acids.[a]

Compound	R_F				Qualitative color reactons			
	IA	BP	BuAc	CiPh	DSA	DNA	DNPH	AmAg
3-MeO-4-HO-								
mandelic acid	0.26	0.12	0.67	0.56	O[i]	Pu	Lt Y[b]	G(slow)
nitrile	[c]	0.42	0.89[d]	-	O[i]	-	None	None
ethyl ester	0.85[e]	0.79	0.92	-	O[i]	-	None	Bl-G (slow)
amide	0.42	0.32	0.58	-	O[i]	Pu	None	G[j]
benzoic acid	0.22	0.78	0.89	-	O[i]	V	None	None
hippuric acid	0.16	0.18	0.74	-	Lt O-R	V	None	None
Vanillin	0.53	0.80	0.90	-	Lt O	-	Lt O	Lt G (slow)[b]
3,4-Dihydroxy-								
benzaldehyde	0.50[f]	0.23	0.84	-	Lt G-Br[g]	Y-O	Y-O	DkG-LtBr
mandelic acid	0.10[f]	0.01	0.49	0.80	Lt Br[g]	Bl-G	O(slow)	Bk→Br
nitrile	[c]	0.05	0.77[d]	-	Lt Br[g]	-	None	Y→G-Br
ethyl ester	0.64[h]	0.21	0.83	-	LtO-Br[g]	-	None	Y-Br ↓ G-Br
benzoic acid	0.06	0.16	0.75	0.46	Lt Br[g]	None	None	Bk
phenylacetic acid	0.06[f]	0.15	0.73	0.81	Lt Br[g]	-	None	Bk

Footnotes to Table I

a/ Solvent systems: IA, isopropyl alcohol-aqueous ammonia-water, 8:1:1, 15 hours; BP, benzene-propionic acid-water, 100:70:5, 3 hours; BuAc, n-nutanol-acetic acid-water, 4:1:1, 15 hours; CiPh, pH 3.0 citrate-phosphate buffer, 2.5 hours (p. 42)

Spray Reagents: DSA, modified diazotized sulfanilic acid (p. 40); DNA, modified diazotized p-nitroaniline (p. 40); DNPH, 2,4-dinitrophenylhydrazine (p. 43); AmAg, ammoniacal silver nitrate (p. 42).

Colors: Lt, light; Dk, dark; Bk, black; Bl, blue; Br, brown; G, grey; O, orange; Pu, purple; R, red; V, violet; Y, yellow.

b/ Require 100 micrograms; 10 micrograms not detected. 3-Methoxy-4-hydroxymandelic does not reduce silver nitrate after run in pH 3.0 buffer.

c/ Completely converted to aldehyde; no other spots observed.

d/ Approximately 6% converted to aldehyde in 12 hours.

e/ Slight trailing indicates minor decomposition (amide formation?).

f/ Spots faintly visible before spray, some trailing (atmospheric oxidation?); when 3,4-dihydroxymandelic acid is chromatographed in large quantities, the decomposition products in IA system give a spot on the chromatogram; this artifact may be inadvertedly mistaken for a metabolite especially in two-dimensional chromatography.

g/ Surrounded by white fringe.

h/ Extensive trailing and abnormal color reaction indicate major decomposition; R_F taken from 100 microgram spot where some ester still intact.

i/ DSA color of 3-methoxy-4-hydroxy compounds varies from orange to red depending on the ratio of compound to reagent.

j/ Slow reduction of silver nitrate after BuAc run and no reduction after BP run.

METABOLIC STUDIES

One path by which endogenous epinephrine and norepinephrine may undergo metabolism has been presented earlier in this work, i.e. via 3,4-dihydroxymandelaldehyde, 3,4-dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid. One or more of the acids (or their derivatives) may be excreted in urine or may undergo further reactions; decarboxylation followed by oxidation, for instance, would produce the corresponding benzoic acids, i.e. protocatechuic acid and vanillic acid, respectively; conjugation, and opening of the ring are other possibilities.

Metabolic experiments were carried out in order to determine the nature and the extent of these reactions. Epinephrine, norepinephrine, 3,4-dihydroxy- and 3-methoxy-4-hydroxymandelic acids were fed to a normal adult human; the urine was collected and processed for metabolites. Normal urine (no ingestion of compounds) and the urine of a patient with pheochromocytoma also were investigated in the same manner. The effectiveness of the extraction procedures was measured with normal urine to which known amounts of metabolites had been added. Conjugated metabolites were sought by acid and by enzymic hydrolysis of urines from which the free acids had been previously removed; the activity of the enzymes was tested on urines which were known to contain the appropriate conjugates, i.e. human urine collected after the ingestion of vanillic acid, and rat urine after the ingestion of 3-methoxy-4-hydroxymandelic acid.

The methods, the results and the significance of these experiments are discussed.

Materials

3,4-Dihydroxy-DL-mandelic acid, 3-methoxy-4-hydroxy-D, L, and DL-mandelic acids were prepared as described earlier in this paper. 3-Methoxy-4-hydroxyhippuric acid (vanilloylglycine) was synthesized by Armstrong.

The bitartrates of l-(-)-epinephrine and of l-(-)-norepinephrine were purchased from Winthrop-Stearns Inc. Chromatographically pure protocatechuic acid was obtained from the Bios Laboratories. Vanillic acid (Reagent grade) was obtained from the K. and K. Laboratories and was recrystallized from water (Norite), m.p. 212-213° Lit. m.p. 211-212° .

"Ketodase", a beef liver β-glucuronidase (5,000 units per 1.0 ml.) was purchased from the Warner-Chilcott Laboratory Division. "Glusulase", a snail preparation containing sulfatase (23,000 units per 0.1 ml.) and β-glucuronidase (18,000 units per 0.1 ml.), was kindly provided by the Endo Laboratories.

All other reagents and solvents employed in the following procedures were commercial products of the highest available purity.

Methods

Basic Extraction Procedure. Varying amounts of compounds were administered orally to an adult human and urine was collected during the following eight hours. Volumes of approximately 250 to 350 ml. were used for the recovery

of the free phenolic acids contained in these urines.

The extractions were carried out by a method which has been described by Shaw, McMillan and Armstrong (67). The urine was acidified to pH 1.5 (Hydrion paper) by the careful addition of concentrated hydrochloric acid, saturated with sodium chloride, and extracted four times with one-third volume portions of ethyl acetate. The combined ethyl acetate extracts were shaken vigorously with successive small volumes of 1 N sodium bicarbonate until the pH of the last three emergent aqueous phases was 7.5 to 8.0. The pooled bicarbonate extracts, usually 20 to 50 ml. volume, were cooled to -10°, acidified to pH 1.5, saturated with sodium chloride and extracted four times with one-quarter volume portions of ethyl acetate. The combined ethyl acetate extracts were diluted with ethyl acetate so that 1 ml. of the final concentrate corresponded to 10 to 15 mg. of the creatinine present in the original urine. The ethyl acetate solution was dried with anhydrous sodium sulfate and stored at -10°.

In order to examine the acids excreted in conjugated forms, the extracted urine was acidified to pH 0.5 and heated in an autoclave one hour at a pressure of 15 pounds per square inch. The liberated metabolites were then extracted by the basic procedure just described. It should be mentioned that, with autoclaved urines, more bicarbonate was needed to attain a pH of 7.5 to 8.0; the volume of the final ethyl acetate concentrate was necessarily greater.

Enzyme Experiments. Enzymic hydrolysis of the phenolic acid conjugates in urine was also performed. The use of enzymes, however, necessitated

changes in the basic procedure.

Only 50 to 100 ml. of urine was processed, in order to conserve the enzyme preparations and to facilitate handling of the urine specimens through the extended treatment. Sodium chloride could not be added to a medium which was to be subjected to enzyme action; compensation was effected by increasing the volume of the ethyl acetate extracts to one double volume portion and three equal volume portions. Three additional extractions were made to avoid carryover of free acids; these last extracts were discarded. After removal of the free acids was completed, the aqueous phase was adjusted to pH 5.5 (Beckman meter) with concentrated aqueous sodium acetate solution. The urine was diluted with water to restore its original volume, since considerable loss of water occurred during the ethyl acetate extraction of the free acids. Two ml. of "Ketodase" (10,000 units of β-glucuronidase) was added for each 30 ml. of urine and a crystal of thymol was placed in the solution to prevent bacterial growth during the subsequent incubation. The container was swept with nitrogen for a few minutes and capped with a rubber stopper. The preparation was thoroughly shaken and was stored at 47° for 16 hours. The urine was then cooled to room temperature, acidified to pH 1.5 and the phenolic acids liberated from the glucuronides were recovered by the salt free method described above.

The resulting aqueous phase was then treated with "Glusulase". The incubation conditions employed here were essentially those found to be satisfactory with "Ketodase"; 0.1 ml. of the enzyme preparation (23,000 units of sulfatase) was used for each 50 ml. of urine. The extraction

of the liberated acids, however, was carried out by the basic procedure, there being no further use for the processed urine.

Clinical Procedure. A simplified extraction procedure was devised for clinical use in the diagnosis of pheochromocytoma. The relation of this pathological condition to the phenolic acids content of urine will be discussed later. Speed and ease of handling were sought.

A volume of urine containing 1 mg. of creatinine was diluted to two ml. with water, acidified to pH 1.5 by the addition of three drops of 3 N hydrochloric acid and shaken vigorously in a 15 ml. centrifuge tube with a two volume portion of ethyl acetate (4 ml.). The ethyl acetate was transferred to another centrifuge tube by means of a capillary pipette. The extraction of the aqueous phase was repeated with a one volume portion of ethyl acetate (2 ml.). The extracts were combined in the second tube and blown to dryness in a current of air; the evaporation of the solvent could be accelerated by placing the tube in a bath at 60°. The residue was washed down from the walls of the tube with a small volume of absolute ethanol and this solvent was carefully removed in the manner just described. The water-free gum in the tube was dissolved in one or two drops of ethanol and the resulting concentrate was chromatographed immediately. The chromatogram was compared, for diagnosis, with one obtained by similar treatment of an equivalent quantity (on a creatinine basis) of normal urine.

Control and Recovery Experiments. Different control experiments, on both the biochemical and extraction procedures, were performed to measure the

effectiveness of the reagents and of the methods employed.

Phenolic acids were extracted from normal urines to establish the endogenous levels of production of epinephrine and norepinephrine metabolites. The basic extraction procedure was used, followed by paper chromatography of the concentrates. The urines used for this purpose were collected on six different days over a period of several months. All collections, including those which were preceded by ingestion of compounds, were made between 7:00 a.m. and 6:00 p.m.; this measure minimized the fluctuations in urinary metabolites which would only reflect changes in output of epinephrine and norepinephrine caused by diurnal variation or possibly by differences in the activities of the subject supplying the urine.

The effectiveness of each of the three recovery methods, i.e. with salt, without salt and the clinical procedure, was tested by adding enough 3-methoxy-4-hydroxymandelic acid to normal urines to obtain concentrations of 0.005, 0.050 and 0.100 mg. per ml.; this range covers the concentrations present in the urines in the metabolic studies. The acid was then extracted and quantitated by paper chromatography.

"Ketodase" was tested on urine collected after the ingestion of 1.0 g. of vanillic acid; this acid is excreted as a glucuronide by the human. The ability of the enzyme to hydrolyze the glucuronide of 3-methoxy-4-hydroxymandelic acid was tested on urine collected from rats which had been fed 250 mg. of the free acid.

Identification of the Metabolites. The nature and the amount of the individual phenolic acids resulting from the metabolism of norepinephrine were determined by paper chromatography of the urine extracts. Ascending chromatography on Whatman No. 1 filter paper was employed. For detection and identification of the phenolic acids, a volume of concentrate corresponding to 1 mg. of urinary creatinine was applied to a 12 by 13 inch sheet at a distance of one inch from the bottom and two inches from the left hand edge. The sheet, stapled into a cylinder along its vertical axis, was placed in a battery jar containing a 0.5 inch layer of an 8:1:1 isopropyl alcohol - concentrated ammonium hydroxide - water solution. The jar was covered with a glass lid and the liquid was allowed to ascend to a line approximately one inch below the top of the paper cylinder, a distance of perhaps 11 inches, in 15 hours. The paper cylinder was removed from the jar, allowed to dry in air, unstapled, a one inch strip of paper was cut off from the left hand edge of the sheet and the new smaller sheet (12" x 12") was once again stapled into a cylinder, this time along its horizontal axis. The new cylinder was hung by a clip and thread in a jar containing a 0.5 inch layer of benzene - propionic acid - water solution (100:70:5). After 30 minutes of equilibration, the cylinder was lowered into the liquid and the solvent was allowed to ascend for a period of three hours. The equilibration step was eliminated from the clinical procedure. The chromatogram was allowed to dry in air at room temperature; in the clinical procedure, drying time could be shortened by placing the chromatogram in an oven at 105° for three minutes.

The sheet was then sprayed with diazotized sulfanilic acid or diazotized p-nitroaniline. The diazotized sulfanilic acid reagent was prepared by mixing one volume of cold 0.2% aqueous sodium nitrite with one volume of a solution of 1 g. of sulfanilic acid in 10 ml. of concentrated hydrochloric acid diluted to 500 ml. with water, allowing this mixture to stand for five minutes in an ice bath, and then adding two volumes of cold 10% aqueous potassium carbonate. The p-nitroaniline reagent was prepared in the same manner except that a 0.1% solution of p-nitroaniline in 1 N hydrochloric acid was substituted for the sulfanilic hydrochloride solution. The concentration of the diazotized amine in these reagents is lower than that recommended in the literature (68, 69); the modification resulted in the elimination of most of the background color on the chromatograms.

The location, the color and the intensity of the spots developed by the reagent were noted and the nature of the compounds thus revealed was determined by comparison with data on the chromatographic behavior of phenolic acids gathered by Armstrong, Shaw and Wall (49). A gross estimate of the quantity of the metabolites could be obtained from these chromatograms.

Quantitation of the Metabolites. More accurate quantitation could be performed by changing the conditions of chromatography to suit the compound measured.

3-Methoxy-4-hydroxymandelic acid was determined in the manner just described, except that the proportions of isopropyl alcohol - ammonium

hydroxide - water were changed from 8:1:1 to 80:2:18. The altered solvent mixture resulted in better resolution of the mandelic acid on two-dimensional chromatograms. The modified diazotized p-nitroaniline reagent also was very helpful in distinguishing 3-methoxy-4-hydroxymandelic acid from the interfering spots of p-hydroxymandelic, m-hydroxyhippuric and 5-hydroxyindoleacetic acids. With diazotized sulfanilic acid, these compounds produce orange, dark yellow, dark yellow and maroon colors, respectively; in contrast, the corresponding p-nitroaniline colors are purple, red, red and pink. For this reason the diazotized p-nitroaniline reagent might be preferred in the clinical procedure; if diazotized sulfanilic acid is used, a large orange spot of 3-methoxy-4-hydroxymandelic acid (pheochromocytoma) might possibly be confused with a large maroon spot given by 5-hydroxyindoleacetic acid (carcinoid), since both compounds appear at nearly the same location on the chromatograms.

The quantitation of 3-methoxy-4-hydroxymandelic acid was carried out by placing on the chromatogram an aliquot of urine extract estimated to contain one microgram of the acid. One, two, three and four microliters of a solution of authentic acid* (0.5 microgram per microliter) were applied to the sheet at distances of 3.5, 4.5, 5.5 and 6.5 inches directly above the spot of unknown. The chromatogram was developed in the modified isopropyl alcohol - ammonium hydroxide - water and in the benzene - propionic acid - water systems. The sheets were sprayed with diazotized p-nitroani-

* Solutions of authentic compounds used for quantitation will be called simply "standard solutions" in the remaining text.

line. The amount of acid in the concentrate was estimated by comparing the unknown to the standard spots.

The quantitation of 3,4-dihydroxymandelic acid was effected by one-dimensional chromatography in a solvent mixture consisting of butanol - acetic acid - water (4:1:1) for 12 hours. The chromatograms were sprayed with ammoniacal silver nitrate. This reagent was prepared by mixing equal volumes of 0.1 N aqueous silver nitrate and 5 N ammonium hydroxide (68). Reference spots of standard solution in the range of 0.25 to 1.5 micrograms, in 0.25 increments, were used.

Protocatechuic acid was estimated on one-dimensional chromatograms run in pH 3.0 McIlvaine citrate-phosphate buffer (70) for 2.5 hours and sprayed with ammoniacal silver nitrate solution. The buffer is prepared by mixing 15.89 ml. of a 0.1 M citric acid solution with 4.11 ml. of a 0.2 M disodium phosphate solution. Reference spots of standard solution in the range of 0.2 to 1.0 microgram, in 0.2 microgram increments, were used.

It should be noted that homoprotocatechuic acid might interfere with the quantitation of protocatechuic acid by one-dimensional chromatography. The absence of the interfering compound from the extracts was established by two-dimensional chromatography in the butanol - acetic acid - water mixture followed by the pH 3.0 citrate-phosphate buffer, after the position of these compounds had been determined previously by chromatographing authentic compounds in the same systems.

Two-dimensional chromatography in the 8:1:1 isopropyl alcohol - ammonium hydroxide - water and the benzene - propionic acid - water systems, as well as in the butanol - acetic acid - water and the pH 3.0 citrate-

phosphate buffer systems, was used for the detection of keto acids in the concentrates. The chromatograms were sprayed with a 0.28% dinitrophenylhydrazine solution in 1 N hydrochloric acid; Armstrong et al. (49) have shown that keto compounds produce characteristic colors with this reagent on chromatograms. Chromatography with the two different pairs of systems was made necessary by the fact that 3,4-dihydroxymandelic and 3-methoxy-4-hydroxymandelic acids give some color with this reagent; they could be identified with certainty, however, by their behavior in all these systems and by spraying similar chromatograms with diazotized sulfanilic acid.

Vanilloylglycine was estimated by the technique employed with 3-methoxy-4-hydroxymandelic acid, except that reference spots of 2.5, 5, 10 and 15 micrograms of authentic compound were used. The quantitation was complicated by the faintness of the spots and, in certain cases, by the interference of relatively high amounts of protocatechuic acid.

The quantitation of vanillic acid was generally done by two-dimensional chromatography in the 8:1:1 isopropyl alcohol - ammonium hydroxide - water and the benzene - propionic acid - water systems. The sheets were sprayed with diazotized p-nitroaniline. Standard spots in the range of 0.5 to 2.5 micrograms, in 0.5 microgram increments, were used. One-dimensional runs in the isopropyl alcohol - ammonium hydroxide - water system were sufficient for the concentrates from urine collected after the ingestion of vanillic acid; the relatively high concentration of vanillic acid in these extracts minimized interference by other compounds with similar R_F values in one-dimensional chromatography.

Results and Discussion

Recovery of 3-Methoxy-4-hydroxymandelic Acid from Urine. An average of 92% (90 to 95%) of the acid added to normal urine was recovered by the basic extraction procedure at all the concentrations tested, i.e. 0.005, 0.050 and 0.100 mg. per ml. A comparable figure of 90% was obtained without the use of sodium chloride. The variations of ± 3% which occurred are well within the limits of accuracy of the technique for the quantitation of this compound (±15%).

The recovery of 3-methoxy-4-hydroxymandelic acid from urine by the clinical procedure varied between 90 and 100%. The lower values were obtained with urines containing more than 20 micrograms of the acid per milligram of urinary creatinine.

Specific recovery experiments for protocatechuic acid, 3,4-dihydroxymandelic acid, vanillic acid and vanilloylglycine were not undertaken. Chromatography of the additional ethyl acetate extracts which were carried out to effect complete removal of the free acids before enzyme treatment of urines, indicated, however, that only very small amounts of these compounds had remained in the urine after the usual extractions. The possible destruction of part of the dihydroxy compounds especially in the sodium bicarbonate extracts must be mentioned; homoprotocatechuic acid, a metabolite of 3,4-dihydroxyphenylalanine has been reported to undergo such decomposition (50).

The autoclaving of the acidified urine at 15 p.s.i. for one hour destroyed 80% of added 3-methoxy-4-hydroxymandelic acid; not a trace of added

3,4-dihydroxymandelic acid could be detected after such treatment.

Normal Excretion of Selected Dihydroxy- and Methoxyhydroxyphenolic Acids. The eight hour daytime excretion of 3-methoxy-4-hydroxymandelic acid by the normal adult human who performed the ingestion experiments in this study varied between 0.8 and 1.0 mg. 3,4-Dihydroxymandelic acid and protocatechuic acid have never been detected in normal urine. The normal output of vanillic acid, both free and combined as a glucuronide, has proved to be very variable from day to day, ranging from amounts too small to detect to well over one milligram in eight hours. This was also true of 3-methoxy-4-hydroxyhippuric acid, its glycine conjugate. This variability is suggestive that most of the vanillic acid usually observed in urine is not endogenous, but probably arises from dietary sources.

Ingestion Experiments. The results of feeding experiments with l-epinephrine, l-norepinephrine, 3,4-dihydroxy- and 3-methoxy-4-hydroxymandelic acids are presented in table II, on page 46.

After the ingestion of 3,4-dihydroxymandelic acid in the quantities indicated in table II, 4 to 9% of the administered compound appeared in urine as 3-methoxy-4-hydroxymandelic acid, 1 to 2% was recovered as protocatechuic acid, and when portions larger than 20 mg. were eaten, 2 to 4% was recovered unchanged. The quantities of vanillic acid and vanilloylglycine found in these urines were often less than one milligram; since, as was reported earlier, more than one milligram of vanillic acid can be found at times in normal urines, it seems that very little of the 3,4-dihydroxymandelic acid is converted to the methoxyhydroxybenzoic acid. However, this does not dispose of the possibility of such conversion; it must be recalled that on certain

TABLE II

Metabolism of l-(-)-Epinephrine, l-(-)-Norepinephrine, and Related Mandelic Acids.[a]

Compound ingested	mg.	Unconjugated acid metabolites in urine[b]						Total recovery %
		3-MeO-4-HO-mandelic		protocatechuic		3-4-di-HO-mandelic		
		mg.	Recovery %	mg.	Recovery %	mg.	Recovery %	
3,4-Dihydroxy-mandelic acid	0	0.9[f]	-	0[e]	-	0	-	
	10	0.9	9	0	0	0	0	9
	20	1.2	6	0.2	1	0	0	7[c]
	50	4.8	9	0.5	1.2	1.2	2.7	13
	100	6.0	6	1.5	1.7	2.2	2.4	10[c]
	100	8.3	8	1.3	1.6	1.9	2.1	12
(2 x 100)[d]	200	11.6	5	3.5	2.1	8.1	4.5	12
(2 x 100)	200	9.8	5	2.4	1.4	2.8	1.4	8[c]
(2 x 250)	500[g]	34.0	6	6.5	1.6	15.2	3.4	11
(2 x 250)	500	22.0	4	6.1	1.5	12.9	2.9	8[c]
3-MeO-4-HO-D-mandelic acid	10	2.3	23	0	0	0	0	23
	100	18.3	18	0	0	0	0	18
3-MeO-4-HO-L-mandelic acid	100	29.4	29	0	0	0	0	29
(-)-Epinephrine	10	0.4	4	0	0	0	0	4
	50[g]	0.5	1	0	0	0	0	1
(-)-Norepinephrine	10	0.4	3	0	0	0	0	3
	50	1.0	2	0	0	0	0	2

Footnotes to Table II

a/ Data obtained on one normal adult human male, except runs covered by note (g).

b/ All values have been corrected for loss due to incomplete recovery and for endogenous output.

c/ These 3,4-dihydroxymandelic acid ingestion runs were carried out three months after the others; they are enzyme runs, extracted without salt.

d/ Ingested in two portions, 30 minutes apart.

e/ Zero stands for undetectable amounts.

f/ Average of six collections.

g/ Ingested by a different normal adult male.

days, the normal production of vanillic acid was negligible and if the mandelic acid was ingested on such a day, a low recovery of vanillic acid might have some significance. Also, since protocatechuic acid is produced when 3,4-dihydroxymandelic acid is ingested, some methylation in the 3-hydroxy position in the manner of homoprotocatechuic acid (50) and of 3,4-dihydroxymandelic acid must have occurred; the biological methylation of larger amounts of protocatechuic acid has been demonstrated by Armstrong (unpublished) and by Booth and collaborators (71). In two instances, i.e. after the ingestion of 100 and 500 mg. of 3,4-dihydroxymandelic acid, a 1 to 2% conversion of this acid to vanilloylglycine seemed to have occurred; this may well have been, but it should be noted again that the protocatechuic acid which was excreted in these experiments, interfered with the normal behavior of vanilloylglycine during chromatography by distorting and pushing the spot away from its usual position on the chromatograms. The study of excretion pattern obtained with ingestions of 3,4-dihydroxymandelic acid was completed by the finding that no keto acids could be detected in the phenolic acids extract; the special measures undertaken to ascertain that the spots produced by the ketone reagent on the chromatograms were caused by the mandelic acids have already been described in the quantitation procedures.

In summary, it has been shown that a significant portion of ingested 3,4-dihydroxymandelic acid is methylated in the 3-hydroxy position, that a small but constant proportion undergoes decarboxylation and oxidation to protocatechuic acid and finally, that smaller amounts of vanillic acid and vanilloylglycine may be formed by methylation of the protocatechuic acid or by decarboxylation and oxidation of the 3-methoxy-4-hydroxymandelic acid.

Ingested 3-methoxy-4-hydroxymandelic acid, D or L, on the other hand, appeared unchanged in the urine in amounts which constituted 18 and 30%, respectively, of the administered dose of 100 mg. No other compound could be detected. The absence of vanillic acid from such urines might suggest that part or all of the protocatechuic acid detected after the ingestion of 3,4-dihydroxymandelic acid arose originally as a chemical impurity in the ingested acid. However, chromatography of the material used for ingestion in the solvent systems employed for the quantitation of protocatechuic and 3,4-dihydroxymandelic acids discounted this possibility: only one spot was produced on the chromatograms by 3,4-dihydroxymandelic acid, and the absence of protocatechualdehyde, protocatechuic acid and 3,4-dihydroxymandelonitrile was demonstrated readily.

When epinephrine and norepinephrine were ingested, only 1 to 4% of the compounds emerged in urine as the 3-methoxy-4-hydroxymandelic acid. Nothing else could be detected. The low recovery makes questionable the positive conclusion that the orally administered amines gave rise to extra 3-methoxy-4-hydroxymandelic acid.

A factor of intestinal absorption might be invoked to explain the low recoveries achieved in these and the other ingestion experiments with the mandelic acids. The relative absence of physical discomfort after the ingestion of 50 mg. of epinephrine and of norepinephrine at the rate of 5 to 10 mg. per 30 minutes during a period of 3.5 hours, would suggest that a rather small portion of the dose penetrated into the blood stream. Richter (72) has reported, among other symptoms, great abdominal distress lasting for several hours after the oral

administration of 30 mg. of epinephrine in one dose.

On the other hand, the lack of discomfort upon ingestion of the amines does not necessarily reflect poor intestinal absorption. It is perhaps more likely that such small doses of low molecular weight compounds were absorbed by the intestine into the portal circulation which leads directly to the liver. In the liver, the compounds could undergo oxidative deamination, decarboxylation, methylation of the phenolic hydroxyl groups and even cleavage of the aromatic ring; only a small proportion would be expected to pass through the liver unchanged. The metabolites would then be secreted partly into the bile and partly into the systemic circulation where ultimately they would reach the kidneys and emerge in urine. In view of these possibilities, the recoveries obtained in the ingestion experiments appear more reasonable since only urinary phenolic acids were determined.

Armstrong, in unpublished work, has shown that about 30% of parenterally administered norepinephrine is excreted in urine in the form of 3-methoxy-4-hydroxymandelic acid. He also demonstrated that five patients with surgically confirmed pheochromocytomas excreted greatly increased amounts of 3-methoxy-4-hydroxymandelic acid and that the excretion levels returned to normal after removal of the tumors. The processing of one of these pathological urines by the clinical procedure confirmed the high content of 3-methoxy-4-hydroxymandelic acid (23 micrograms per mg. of creatinine; normal level, 1 to 3 micrograms). Lastly, 3-methoxy-4-hydroxy-D(-)-mandelic acid has been isolated from a pheochromocytoma urine and characterized by Armstrong et al. (73).

The presence of 3-methoxy-4-hydroxy-D(-)-mandelic acid in urine and

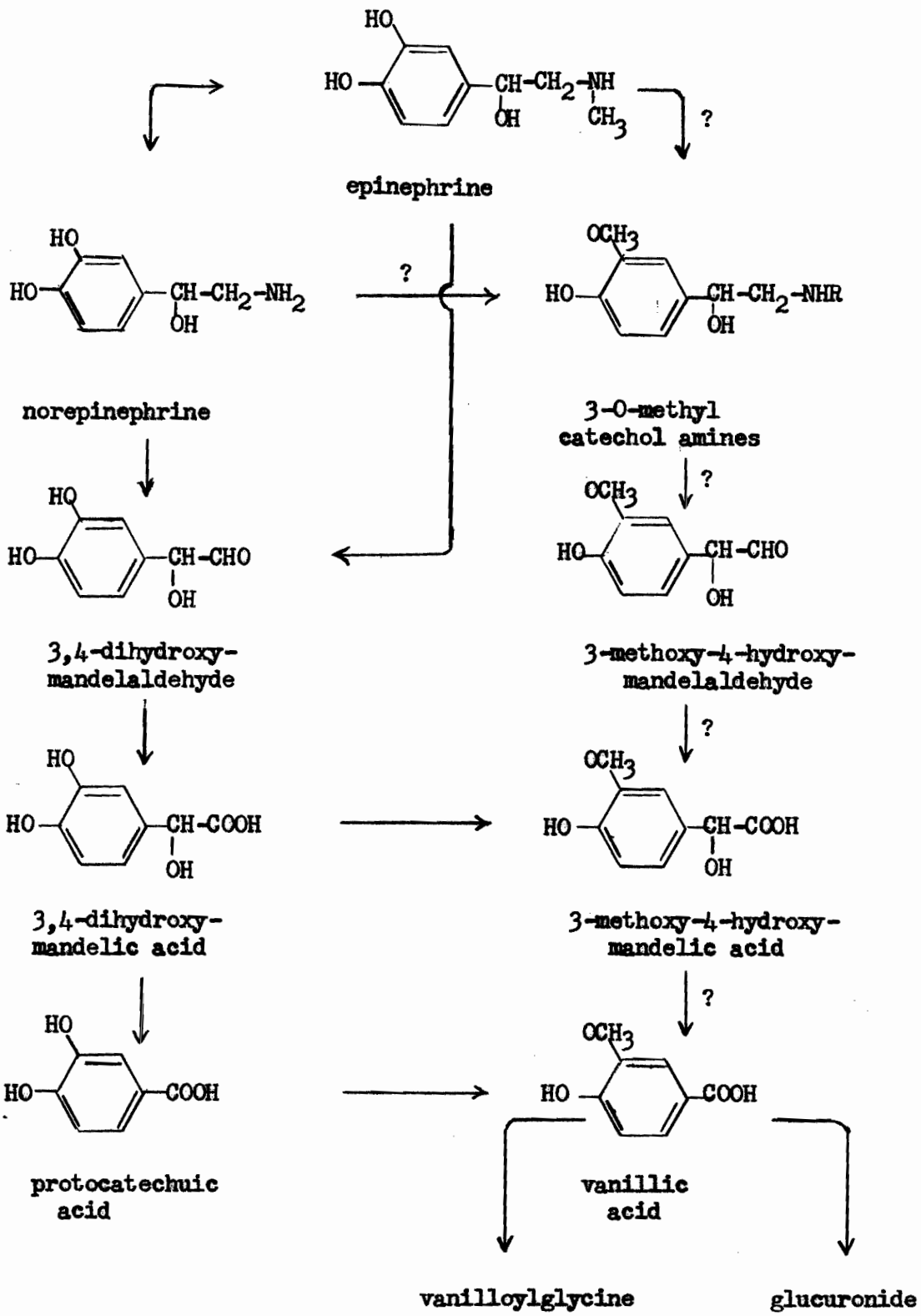
the finding that only 18% of this compound is recovered from the urine after ingestion might suggest that the recovery of ingested 3,4-dihydroxymandelic acid as 3-methoxy-4-hydroxymandelic acid which was reported earlier (4 to 9%) is not a true measure of the importance of this metabolic pathway; 20 to 40% of the ingested 3,4-dihydroxymandelic acid might actually be converted to the methylated compound.

These data are offered in support of the degradation of norepinephrine by way of oxidative deamination to the aldehyde, oxidation to 3,4-dihydroxymandelic acid, followed by methylation or decarboxylation to 3-methoxy-4-hydroxymandelic acid and to protocatechuic acid, respectively. This sequence of reactions is illustrated on the next page.

It is likely also that 3-methoxy-4-hydroxymandelic acid is a metabolite of epinephrine, since, as it was mentioned earlier, in the literature review, both epinephrine and norepinephrine are substrates for amine oxidase and the distribution of radioactive compounds in the urine of rats has been shown to be similar after administration of C^{14}-labelled epinephrine and norepinephrine (74).

Although it has been established that 3-methoxy-4-hydroxymandelic acid can arise by methylation of 3,4-dihydroxymandelic acid, which is known to be formed from the catechol amines by the action of amine oxidase and aldehyde oxidase, it should be noted that the methylation of the 3-hydroxy group of epinephrine and norepinephrine might possibly precede oxidative deamination. In this case, methylation would provide a direct means of inactivation of the amines; this would make this reaction one of great physiological importance. The remarkable difference in recovery of 3-

A Metabolic Path of Epinephrine and Norepinephrine



methoxy-4-hydroxymandelic acid following the parenteral injection of norepinephrine and oral ingestion of 3,4-dihydroxymandelic acid tends to support this latter pathway. In parenteral administration experiments, the role of the liver would be weakened and the production of non-phenolic acid metabolites minimized. It has not been possible to detect methylated catechol amines by the methods used in these studies; the need for tissue experiments is indicated.

The search for conjugated metabolites of 3,4-dihydroxymandelic and of 3-methoxy-4-hydroxymandelic acids in human urine was fruitless. Interference of some substance in human urine with the action of the enzyme might have accounted for the negative results. This idea was tested on human urine collected after the ingestion of 1000 mg. of vanillic acid and on the urine of rats which had been fed 250 mg. of 3-methoxy-4-hydroxymandelic acid. In the case of vanillic acid, 148 mg. or 15% of the ingested compound was recovered from the urine in the free form and another 159 mg., or 16%, was obtained after treatment with "Ketodase". The possibility that the enzyme might be inhibited by some factor present in 3,4-dihydroxymandelic acid urine remains; "Ketodase" liberated only 4.5 mg. of vanillic acid from a mixture of urine containing 7 mg. of vanillic acid as the glucuronide with an equal volume of 3,4-dihydroxymandelic acid urine.

In rat urine, it was found that most of the ingested 3-methoxy-4-hydroxymandelic acid was excreted as the glucuronide and that this conjugate is hydrolyzed by "Ketodase".

Enzymic hydrolysis of 3,4-dihydroxymandelic acid urine with "Glusulase", before or after treatment with "Ketodase", failed to reveal the presence of sulfate conjugates. The particular β-glucuronidase - sulfatase preparation used, however, did liberate the small amounts of vanillic acid mentioned earlier and has been shown to liberate steroids from their conjugates in human urine. Still, enzyme specificity may be involved; a recent review on sulfatases supports this idea strongly (75).

It might be appropriate to close this discussion on the metabolism of epinephrine and norepinephrine with a remark on their configuration. It has been shown that it is the levorotatory form of these catechol amines which is physiologically active. The isolation from urine and the characterization of 3-methoxy-4-hydroxy-(-)-mandelic acid was also mentioned. Remembering then that the levorotatory mandelic acid was shown to have the D configuration by the enzymic hydrolysis of the DL-amide with L-leucine aminopeptidase, it appears likely that the natural isomers of epinephrine and norepinephrine are molecules of the D configuration. The possibility of inversion during enzymic action in the process of metabolism cannot be as yet excluded, however.

SUMMARY

1. The literature concerning norepinephrine has been reviewed briefly in respect to the discovery, the biosynthesis and the metabolism of the compound. Special attention was paid to the degradation of the catechol amines by amine oxidase.

2. Gardner and Hibbert's synthesis of 3-methoxy-4-hydroxymandelic acid from vanillin via the cyanohydrin and the ester has been considerably improved. The method was adapted with fair success to the preparation of 3,4-dihydroxymandelic acid; no satisfactory synthesis had been reported for this compound.

3. Synthetic 3-methoxy-4-hydroxymandelic acid was resolved by means of its cinchonine salts; the enantiomorphs were regenerated from the crude salts and characterized. A small amount of pure cinchonine 3-methoxy-4-hydroxy-(-)-mandelate was prepared.

4. The configuration of the isomers of 3-methoxy-4-hydroxymandelic acid was established by enzymic hydrolysis of the DL-amide with L-leucine aminopeptidase. 3-Methoxy-4-hydroxy-D(-)-mandelamide was obtained in pure form by this process and was characterized. The isomers of the acid were found to be 3-methoxy-4-hydroxy-L(+)-mandelic acid and 3-methoxy-4-hydroxy-D(-)-mandelic acid.

5. 3-Methoxy-4-hydroxymandelic acid has been shown to be a metabolite of 3,4-dihydroxymandelic acid, norepinephrine and possibly, epinephrine.

The catechol amines are first converted to 3,4-dihydroxymandelaldehyde by the action of amine oxidase. The aldehyde is further oxidized to 3,4-dihydroxymandelic acid. The acid is then methylated and decarboxylated to give respectively, 3-methoxy-4-hydroxymandelic acid and protocatechuic acid. These compounds, with the exception of the aldehyde and protocatechuic acid, were ingested and the phenolic acids metabolites were extracted from the resulting urines and determined by paper chromatography. Further evidence for the metabolic path was provided by the detection, isolation and characterization of 3-methoxy-4-hydroxymandelic acid from the urine of patients with pheochromocytoma and by the finding of increased amounts of urinary 3-methoxy-4-hydroxymandelic acid after the parenteral injection of norepinephrine.

6. A clinical procedure for the diagnosis of pheochromocytoma has been developed. It is based on the fact that patients in this condition excrete increased amounts of 3-methoxy-4-hydroxymandelic acid. The increased output of the compound is observed readily when two-dimensional chromatograms of phenolic acids concentrates from pathological urines are compared to similar chromatograms prepared from equivalent amounts (1 mg. of urinary creatinine) of normal urine. The greater stability of 3-methoxy-4-hydroxymandelic acid, as compared to that of the catechol amines, eliminates the special precautions and involved techniques required in the determination of the latter compounds.

7. In the course of testing the β-glucuronidase used for the detection of conjugated metabolites, it was shown that ingested vanillic

acid is excreted in human urine as the free acid, as the glucuronide and as the substituted hippuric acid. Vanillic acid and its glucuronide were present in equal amounts; the quantity of vanilloylglycine was not determined.

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